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# Changes in $\beta_1$- and $\beta_2$-adrenergic receptor mRNA levels in brown adipose tissue and heart of hypothyroid rats

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The aim of the present work was to study the effect of hypothyroidism on the expression of the β-adrenergic receptor (β-AR) in interscapular brown adipose tissue and heart. The total density of plasma membrane β-AR per tissue is decreased by 44% in hypothyroid rat interscapular brown adipose tissue and by 55% in hypothyroid rat heart compared with euthyroid controls. The effects of hypothyroidism on the density of both $\beta_1$- and $\beta_2$-AR subtypes were also determined in competition displacement experiments. The densities of $\beta_1$- and $\beta_2$-AR per tissue are decreased by 50% and 48% respectively in interscapular brown adipose tissue and by 52% and 54% in the heart. Northern blot analysis of poly(A)$^+$ RNA from hypothyroid rat interscapular brown adipose tissue demonstrated that the levels of $\beta_1$- and $\beta_2$-AR mRNA per tissue are decreased by 73% and 58% respectively, whereas in hypothyroid heart, only the $\beta_1$-AR mRNA is decreased, by 43%. The effect of hypothyroidism on the $\beta_1$-AR mRNA is significantly more marked in the interscapular brown adipose tissue than in the heart. These results indicate that β-AR mRNA levels are differentially regulated in rat interscapular brown adipose tissue and heart, and suggest that the decrease in β-AR number in interscapular brown adipose tissue and heart of hypothyroid animals may in part be explained by a decreased steady-state level of β-AR mRNA.

## INTRODUCTION

Brown adipose tissue (BAT), the main effector of cold- and diet-induced thermogenesis in rodents [1,2], is under the control of the sympathetic nervous system [3], which acts through β-adrenergic receptors (β-ARs) [4]. Both $\beta_1$- and $\beta_2$-AR subtypes coexist in BAT [5,6], the $\beta_1$-AR being more abundant [6]. Thyroid hormones have been shown to play a permissive role in cold-induced adaptative changes in the BAT [7,8]. Consistent with this notion is the finding of a decreased metabolic response to sympathetic stimulation in the BAT of hypothyroid rats, which is associated with a decrease in β-AR number in this tissue [9].

Cardiac rate and contractility are also under the control of catecholamines, which act through β-ARs. Both $\beta_1$- and $\beta_2$-AR subtypes coexist in the myocardium of the rat, the $\beta_1$-AR being much more abundant [10]. It is well documented that thyroid hormones influence the response of the heart to catecholamines. In hyperthyroid animals, an increase in cardiac sensitivity to adrenergic stimulation has been described and is associated with an increase in β-AR number [11–14]. In hypothyroid animals, on the other hand, a decreased adrenergic response is observed and is associated with a decrease in the number of β-ARs [11,13,15–17], which can be restored to normal values by thyroxine administration [15,16].

The cloning of genes encoding β-AR subtypes [18–20] has allowed the study of receptors at the level of gene transcription, by monitoring changes in the levels of receptor-specific mRNAs. In various tissues, $\beta_1$- and $\beta_2$-AR mRNAs have been shown to be distinct based on their size, and the proportion of each subtype mRNA has been found to correlate well with the proportion of each β-AR determined by ligand-binding analysis [20]. Most of the studies performed to date on transcriptional regulation of β-ARs have dealt only with the $\beta_2$-AR. $\beta_2$-AR mRNA was found to be increased by glucocorticoid treatment in cultured DDT$_1$ MF-2 hamster smooth muscle cells and in S49 mouse lymphoma cells [21,22], and decreased by exposure to β-AR agonists [23–25]. Recently, a comparison of the expression of $\beta_1$- and $\beta_2$-ARs was made in differentiating 3T3-L1 cells: cell differentiation was reported to induce a decrease and an increase respectively in $\beta_1$- and $\beta_2$-AR mRNA [26]. Only one study has been performed *in vivo*, which demonstrated that the level of $\beta_2$-AR mRNA in rat ventral prostate is regulated by testosterone [27]. No studies to date have examined the possible effects of thyroid hormones on the modulation of $\beta_1$- and $\beta_2$-AR subtype mRNAs either *in vitro* or *in vivo*.

The aim of this work was to study the effect of thyroid hormones on the density of the β-ARs and on the levels of β-AR mRNA in the interscapular BAT (IBAT). The effects of thyroid hormones on long-term cold-induced IBAT growth and mitochondrial changes occur normally in the presence of only small amounts of thyroxine in thyroidectomized rats and do not occur in intact rats treated with large amounts of thyroxine. Thus thyroid hormones do not appear to be involved other than in a permissive way in the response of the BAT to catecholamines [7,8]. Consequently, the model chosen in the present work to study the effect of thyroid hormones on the density of β-ARs and on the levels of β-AR mRNA in IBAT was hypothyroidism. Comparison was made with the heart to detect possible differences in the responses of these two tissues to changes in thyroid status.

Abbreviations used: BAT, brown adipose tissue; β-AR, β-adrenergic receptor; IBAT, interscapular BAT; MMI, 2-mercapto-1-methylimidazole; $IC_{50}$, concn. causing half-maximal inhibition of binding.

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## MATERIALS AND METHODS

### Materials

All organic and inorganic chemicals were of analytical or molecular biology grade and were purchased from Merck (Darmstadt, Germany), Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland), Gibco BRL (New York, NY, U.S.A.), Pharmacia (Uppsala, Sweden) or Boehringer (Mannheim, Germany). [$^{125}$I]Iodocyanopindolol (2000 Ci/mmol), [$\gamma$-$^{32}$P]ATP (1000–3000 Ci/mmol), [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) and Gene Screen Plus membrane filters were purchased from NEN (Boston, MA, U.S.A.). ICI 118551 was a gift from ICI (Macclesfield, Cheshire, U.K.).

### Animals

Sprague–Dawley male rats were kept at room temperature (22 °C) with 12 h of illumination per day and were fed *ad libitum* with Provimi Lacta chow (Cossonay, Switzerland). Chemical hypothyroidism was induced by the addition of 2-mercapto-1-methylimidazole (MMI), 1 g/litre, to the drinking water for a period of at least 4–5 weeks, starting when the rats were 6 weeks of age. Hypothyroid status was assessed by measuring plasma thyroid-stimulating hormone and thyroxine concentrations. Rats were found to become hypothyroid after 1 week of treatment. Control rats were age-matched with hypothyroid rats. All preparations were pooled samples of tissues from 2–4 animals. The IBAT and the heart were rapidly excised. IBAT plasma membranes were prepared as described previously [28] and heart crude plasma membranes were isolated using the technique described by McMurchie *et al.* [29]. Protein concentration was determined by the method of Lowry *et al.* [30]. The tissues to be used for RNA isolation were rapidly frozen in liquid nitrogen and stored at −70 °C.

### $\beta$-AR radioligand-binding studies

In radioligand-binding studies, membranes were incubated for 30 min at 37 °C in Tris/HCl (50 mM) (pH 7.4)/$MgCl_2$ (10 mM) (total volume 0.5 ml) containing 25–400 pM-[$^{125}$I]iodocyanopindolol. The binding of the radioligand to the membranes was determined by filtration using a Brandel M-24 R apparatus. Specific binding was defined as the difference between the total binding obtained in the absence of competing ligand and the non-specific binding obtained in the presence of 100 $\mu$M-(−)-isoprenaline. Each assay was performed in duplicate. A series of competition experiments was performed using [$^{125}$I]iodocyanopindolol (50 pM) and increasing concentrations (1, 2, 5, 10, 20, 50, 200, 500 nM and 1, 10, 100 $\mu$M) of the $\beta_2$-AR subtype selective antagonist ICI 118551. The quantitative parameters ($K_d$ and $B_{max.}$) of specific [$^{125}$I]iodocyanopindolol binding were determined by Scatchard-plot analysis [31] using the LIGAND program [32]. The percentage of $\beta_1$- and $\beta_2$-AR subtypes present in each tissue was determined from ICI 118,551 displacement curves by Hofstee analysis.

### RNA isolation

Total IBAT and heart RNA was isolated by the caesium/trifluoroacetic acid gradient method of Okayama *et al.* [33]. Briefly, the frozen tissues were pulverized in liquid nitrogen, transferred directly to 19 ml of 5.5 M-guanidium isothiocyanate/25 mM-sodium citrate/0.5% lauryl sarcosine/200 mM-$\beta$-mercaptoethanol at pH 7.0 and homogenized with a Polytron homogenizer, setting 5, for 20 s. The homogenate was centrifuged at 350 *g* for 5 min and the supernatant was layered over 17 ml of caesium/trifluoroacetic acid at a density of 1.5 g/ml containing 0.1 M-EDTA at pH 7.4, and centrifuged at 110000 *g* for 24 h. The poly(A)$^+$ RNA was isolated from the total RNA by the method described by Aviv & Leder [34] using prepacked oligo(dT)–cellulose columns from Pharmacia (Uppsala, Sweden). The yields of poly(A)$^+$ RNA varied between 2 and 4%.

### RNA blot hybridization

Total RNA (20 $\mu$g) or poly(A)$^+$ RNA (15 $\mu$g) was electrophoresed in an agarose gel containing formaldehyde as described by Lehrbach *et al.* [35] and transferred to Gene Screen Plus membranes by capillary blotting. A human genomic $\beta_1$-AR DNA probe (D. A. Robinson & J. C. Venter, unpublished work), a rat heart $\beta_2$-AR cDNA probe [36], and a mouse pAL 41 recombinant $\beta$-actin probe (gift from Dr. Patrick Iynedjian, Institut de Biochimie Cliniquè, Centre Médical Universitaire, Genève) were labelled by random priming with [$\alpha$-$^{32}$P]dCTP to a specific radioactivity of approx. $1 \times 10^9$ d.p.m./$\mu$g of DNA. The oligo(dT)$_{12-18}$ probe obtained from Pharmacia was labelled by $T_4$ polynucleotide kinase with [$\gamma$-$^{32}$P]ATP. RNA blots were hybridized overnight at 42 °C in 45% formamide and 4 × SSC (0.6 M-NaCl/0.06 M-sodium citrate) and then washed successively in a solution of 0.1 × SSC/0.1% SDS at 55 °C and 68 °C for 15 min and exposed to Kodak X-AR films at −70 °C. Size estimates of the RNA species were established by comparison with an RNA ladder. Densitometric analysis of autoradiograms was performed with a high-resolution laser-beam densitometer. Student's unpaired *t* test was used to determine statistical significance.

## RESULTS

Fig. 1 illustrates the radioligand-binding studies performed on plasma membranes prepared from IBAT or heart from control and hypothyroid rats. Scatchard analysis of saturation isotherms indicated the presence of a single population of binding sites in both tissues. The calculated $B_{max.}$ values were 191 ± 11 and 183 ± 52 fmol of ligand bound/tissue in IBAT and heart respectively of control rats (means ± S.E.M. of three experiments).

In order to quantify the percentages of $\beta_1$- and $\beta_2$-AR receptor subtypes present in each of these tissues, competition displacement of [$^{125}$I]iodocyanopindolol binding to IBAT and heart $\beta$-ARs was performed using the $\beta_2$-AR-selective antagonist ICI 118551. In both tissues, the competition displacement curves were characterized by a slope of less than 1.0, suggesting the presence of more than one population of binding sites. Computer analysis of ICI 118551 binding to IBAT and heart revealed the presence of two affinity states which presumably represented $\beta_1$-AR [$IC_{50}$ (concn. causing half-maximal inhibition of binding) 700 and 196 nM in IBAT and heart respectively] and $\beta_2$-AR ($IC_{50}$ 43 and 5 nM in IBAT and heart respectively) in each of these tissues. The percentages of $\beta_1$- and $\beta_2$-AR were calculated by Hofstee analysis and were found to be 72 ± 3% and 28 ± 3% ($n = 4$) respectively in IBAT and 73 ± 8% and 27 ± 8% ($n = 4$) respectively in heart. These values are in very good agreement with the data of Levin & Sullivan [6] and with those of Minneman *et al.* [10] on the density of $\beta$-AR subtypes in IBAT and heart respectively.

In both IBAT and heart of hypothyroid rats there was a decrease in the total density of $\beta$-AR per tissue of 44 ± 4% ($P < 0.005$, $n = 3$) and 55 ± 3% ($P < 0.005$, $n = 3$) respectively as compared with controls. There was no change, however, in the $K_d$ of the $\beta$-AR for the radioligand [$^{125}$I]iodocyanopindolol (IBAT, 180 ± 36 and 208 ± 67; heart, 127 ± 37 and 123 ± 38 pM in control and hypothyroid rats respectively). The effect of hypothyroidism on the density of both $\beta_1$-and $\beta_2$-AR subtypes was also determined in competition displacement experiments. As

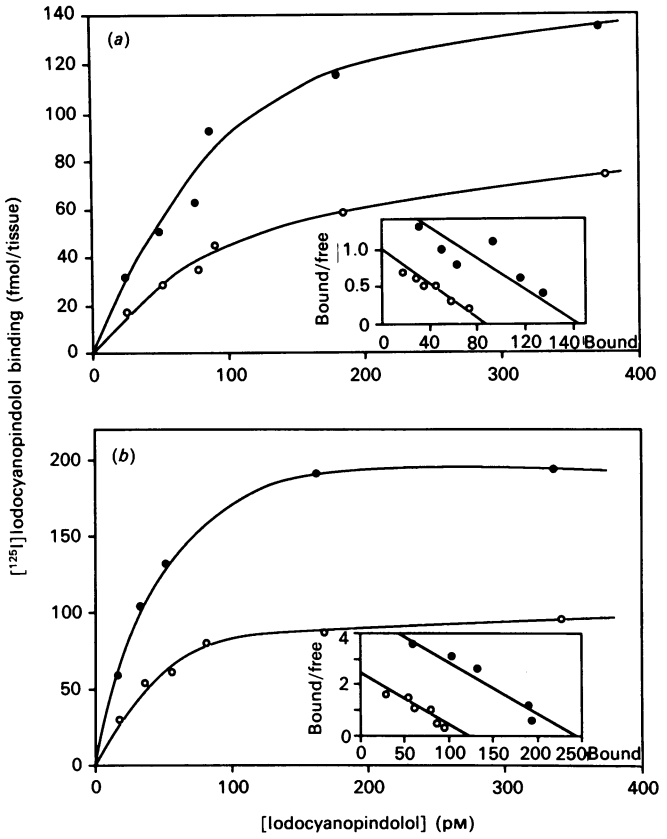


**Fig. 1. Specific binding of [$^{125}$I]iodocyanopindolol to IBAT (*a*) and heart (*b*) plasma membranes from control (●) and MMI-treated hypothyroid (○) rats as a function of increasing concentrations of the ligand**

The results illustrated are from one representative experiment and are expressed, accounting for the amount of plasma membrane proteins recovered from one tissue, in fmol of ligand bound per tissue. Inset: Scatchard analysis of the data.

summarized in Table 1, hypothyroidism resulted in 50% and 48% decreases respectively in the density of $\beta_1$- and $\beta_2$-ARs in IBAT and 52% and 54% decreases respectively in the density of $\beta_1$- and $\beta_2$-ARs in the heart. Thus, in both tissues, there are comparable decreases in the density of each receptor subtype in hypothyroidism.

Consistent with ligand-binding data were our findings that two species of $\beta$-AR mRNA coexist in IBAT and heart: a 3.1 kb species that hybridizes with the $\beta_1$-AR probe and a 2.3 kb species that hybridizes with the $\beta_2$-AR probe (Fig. 2). In both tissues, the $\beta_2$-AR probe slightly cross-reacts with the 18 S rRNA. Thyroid hormones are known to act in target tissues to modulate the transcription of specific genes. Since it has been shown that there is an increase in the cardiac mRNA level in hyperthyroid rat [37], it was of interest to us to first determine whether hypothyroidism produced a change in the total mRNA present in IBAT and heart. Northern blots of total RNA from these tissues in control and treated animals were hybridized with a $^{32}$P-labelled recombinant $\beta$-actin probe, since actin is generally considered to be a good marker of total mRNA, or a [$\gamma$-$^{32}$P]oligo(dT) probe (Fig. 3). Densitometric analysis of the autoradiograms revealed that the amount of $\beta$-actin mRNA is decreased in hypothyroidism by 37% ($P < 0.005$, $n = 5$) in the IBAT and the amount of $\alpha$-actin mRNA is decreased by 47% ($P < 0.005$, $n = 5$) in the heart as compared with controls. These data suggest that there may be a decrease in the total amount of mRNA in hypothyroid IBAT and heart. Analysis of the same autoradiograms which were hybridized with [$\gamma$-$^{32}$P]oligo(dT) also indicated that the amount of mRNA is decreased in hypothyroidism, by 29% ($P < 0.001$; $n = 5$) and 23% ($P < 0.05$; $n = 4$) in IBAT and heart respectively as compared with controls. Since the actin mRNA was more depressed in hypothyroid rat tissues than the total mRNA measured with the oligo(dT) probe, it is suggested that the level of actin mRNA may be selectively modulated by thyroid hormones. Therefore the [$\gamma$-$^{32}$P]oligo(dT) signal was used as the marker of the level of cellular mRNA.

In order to examine whether the hypothyroid state was associated with changes in the level of mRNA encoding $\beta_1$- and $\beta_2$-ARs in IBAT and heart, we utilized Northern blot analysis to quantify receptor-specific mRNAs in these tissues. Northern blots of poly(A)$^+$ mRNA isolated from control or hypothyroid rats were hybridized with $\beta_1$- and $\beta_2$-AR probes. In order to account for the change in total mRNA recovered from each tissue in control versus hypothyroid animals, we expressed the changes in the level of receptor-specific mRNA per IBAT or per heart (Table 1). These values were calculated in the following way. The densitometric analysis values were first normalized for the actual amount of poly(A)$^+$ RNA on the membrane, which was determined by using the corresponding [$\gamma$-$^{32}$P]oligo(dT) signal. It was thus possible to calculate the changes in the amounts of $\beta_1$- or $\beta_2$-AR mRNA in a known amount of poly(A)$^+$ in tissues of hypothyroid rats as compared with controls. The changes per tissue were calculated by accounting for changes in the amount of poly(A)$^+$ recovered from the total RNA of one tissue. In the IBAT, the levels of $\beta_1$- and $\beta_2$-AR mRNA per tissue were decreased by 73% and 58% respectively in hypothyroid rats as compared with control animals. In the heart the level of $\beta_1$-AR mRNA was decreased by 43%, whereas the level of $\beta_2$-AR mRNA was decreased, but not significantly (by 18%). The effects of hypothyroidism on the $\beta_1$- and $\beta_2$-AR mRNA are significantly ($P < 0.05$ and 0.01 respectively) more marked in the IBAT than in the heart. The correlation between the decreases in

**Table 1. $\beta_1$- and $\beta_2$-AR numbers and $\beta_1$- and $\beta_2$-AR mRNA levels expressed per tissue in the IBAT and heart of hypothyroid rats and controls**

The results are the changes in binding values or mRNA levels per tissue, accounting for the amount of plasma membrane proteins recovered from one tissue and for the amount of poly(A)$^+$ recovered from the total RNA of one tissue respectively. The values are the means ± S.E.M. of the numbers of experiments in parentheses, expressed as percentages of control values. *$P < 0.05$; **$P < 0.01$ versus respective controls.

| | [$^{125}$I]Iodocyanopindolol binding (% of control) | | mRNA level (% of control) | |
|---|---|---|---|---|
| | $\beta_1$ | $\beta_2$ | $\beta_1$ | $\beta_2$ |
| IBAT | 50.5 ± 1.4 (3)** | 52.4 ± 10.3 (3)* | 27.4 ± 10.9 (5)** | 42.5 ± 9.5 (4)** |
| Heart | 48.0 ± 1.8 (3)** | 46.0 ± 2.1 (3)** | 56.5 ± 5.0 (4)** | 82.3 ± 7.7 (4) |

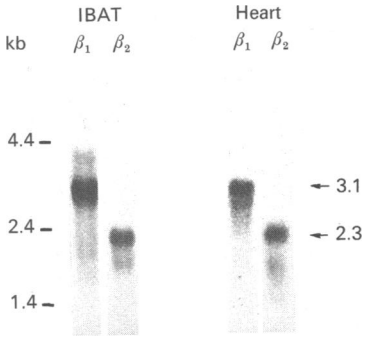


**Fig. 2. $\beta_1$- and $\beta_2$-AR mRNA signals in rat IBAT and heart**

Poly(A)$^+$ RNA (15 $\mu$g) was electrophoresed, transferred and hybridized with $^{32}$P-labelled $\beta_1$- and $\beta_2$-AR probes as described in the Materials and methods section. A representative autoradiogram is shown. The positions of marker RNAs are given in kilobases (kb).

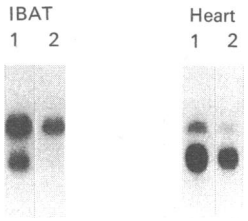


**Fig. 3. Steady-state levels of $\beta$-actin mRNA in the IBAT and the heart of control (lane 1) and hypothyroid (lane 2) rats**

Total RNA (20 $\mu$g) was electrophoresed, transferred and hybridized with a $^{32}$P-labelled recombinant $\beta$-actin probe as described in the Materials and methods section. A representative autoradiogram is shown. The upper signal is $\beta$-actin (2.1 kb); the lower signal is $\alpha$-actin (1.6 kb).

the densities of $\beta$-AR subtypes measured with ligand binding and those of their respective mRNAs is quite good, with the exception of the $\beta_2$-AR in the heart, which was decreased by 54% although there was little change in the level of $\beta_2$-AR mRNA.

## DISCUSSION

Thyroid hormones are known to exert a number of physiological effects in target tissues via nuclear receptors that stimulate or inhibit the transcription of specific genes [38,39]. The effects of thyroid hormones on $\beta$-AR-mediated responses and receptor numbers in rat IBAT and heart are well-documented [7–9, 11–17], suggesting that thyroid hormones may modulate $\beta$-AR gene expression. Thus the aim of this study was to examine the effects of changes in thyroid status on the levels of $\beta$-AR and receptor-specific mRNA in these tissues.

Previous studies have demonstrated that the density of the $\beta$-ARs in IBAT and heart from hypothyroid rats is significantly lower than in control animals [9,11,13,15–17]. The results of our radioligand-binding studies confirm these earlier reports and indicate that the expression of $\beta$-receptors in IBAT and heart is decreased by 44% and 55% respectively in hypothyroid animals.

Analysis of $\beta$-AR radioligand-binding data from rat IBAT and heart reported earlier [5,6] and in this study suggest the presence of both $\beta_1$- and $\beta_2$-AR subtypes in these tissues, the $\beta_1$-AR being more abundant. Consistent with this hypothesis are the results in this study, which demonstrate that both $\beta_1$- and $\beta_2$-AR-specific mRNAs are expressed in IBAT and heart. The size of the $\beta_2$-AR mRNA (2.3 kb) found in the present study is similar to that previously described in rat heart [20], while that of the $\beta_1$-AR mRNA (3.1 kb) is slightly higher than that of 2.5 kb described in rat heart [20].

Northern blot analysis of poly(A)$^+$ RNA from control and hypothyroid rats demonstrates that hypothyroidism is associated with a specific decrease in the level of mRNA for both $\beta_1$- and $\beta_2$-ARs in IBAT. These data are in contrast with our findings in the heart, which suggest that hypothyroidism is associated with a decrease in the level of $\beta_1$-AR specific mRNA only. Our results further show that in both IBAT and heart from hypothyroid rats, there is a decrease in the amount of total mRNA present in a known quantity of total RNA. It might be argued that this decrease in mRNA is only apparent, due to an increase in other RNA species. The amount of total RNA recovered per g of IBAT or heart tissue is the same in hypothyroid and control rats (results not shown), and suggests therefore that the observed decrease in mRNA is real. It has previously been demonstrated that in rats made hyperthyroid with L-tri-iodothyronine there is an increase in the cardiac mRNA level and an increase in the protein synthesis rate [37]. The results of the present study suggest that the opposite may be true in hypothyroidism, and extend these findings to IBAT. In IBAT the decreases in the amounts of $\beta_1$- and $\beta_2$-AR mRNA per tissue observed in hypothyroidism correlate well with the decrease in the number of the corresponding receptors (Table 1), suggesting that decreased levels of $\beta_1$- and $\beta_2$-AR mRNA in this tissue may be responsible for the observed changes in receptor expression. The same seems to be true for the $\beta_1$-AR in the heart. The decrease in the $\beta_2$-AR in the heart, however, apparently cannot be explained by a decrease in its mRNA.

Experimental evidence supports the notion that thyroid-hormone–receptor complexes bind to *cis*-acting DNA sequences (thyroid hormone response elements) in the 5′-flanking region of a gene to influence the rate of gene transcription [38,39]. Computer analysis of the 5′ untranslated region of the human $\beta_2$-AR as described by Emorine *et al.* [40] has in fact revealed the presence of three putative thyroid hormone response elements: a 16 bp sequence described by Glass *et al.* [41] from bases −686 to −671 with four mismatches, and a 14 bp sequence associated with the rat growth hormone gene described by Wight *et al.* [42] from bases −695 to −682 with two mismatches and from bases −747 to −735 with three mismatches. Each subtype of $\beta$-AR receptor is most likely encoded in both IBAT and heart by the same gene possessing the same 5′ thyroid hormone response elements. However, our results demonstrate a tissue-specific regulation of $\beta$-AR subtypes in hypothyroidism. These data suggest that IBAT and heart may contain distinct thyroid hormone receptors with different specificities [43] or different *trans*-acting factors that are responsible for the observed differences in regulation of $\beta_1$- and $\beta_2$-AR genes [38,39]. While we have not, as yet, identified the thyroid hormone response elements involved, this is obviously an area for future study.

It has also been demonstrated that the levels of various hormones are affected by the thyroid status. A decreased rate of cortisol production has been reported in hypothyroidism [44]. This decrease should not, however, account for the observed decrease in IBAT and heart $\beta$-AR density. It has been shown, indeed, that in adrenalectomized animals the density of the $\beta$-AR is unchanged in the IBAT [45] and is higher than in control animals in the heart [46]. An increased sympathetic activity has also been described in the IBAT [47] and in the heart [48] of hypothyroid animals. This increase might, by inducing a de-

sensitization, play a role in the decrease in $\beta$-AR density in both IBAT and heart.

The results of this study indicate that the decrease in $\beta$-AR number in the IBAT and heart of hypothyroid animals may in part be explained by a decreased steady-state level of $\beta$-AR mRNA.

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